Title

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Journal

The Journal of craniofacial surgery, 25(4)

ISSN

1049-2275

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Publication Date

2014-07-01

DOI

10.1097/scs.0000000000000755

Peer reviewed

Differentiated Adipose-Derived Stem Cell Cocultures for Bone Regeneration in Polymer Scaffolds In Vivo

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Abstract: Critical-sized bone defects can lead to significant morbidity, and interventions are limited by the availability and donor-site morbidity of bone grafts. Polymer scaffolds seeded with cells have been explored to replace bone grafts. Adipose-derived stem cells have shown great promise for vascularization and osteogenesis of these constructs, and cocultures of differentiated stem cells are being explored to augment vessel and bone formation. Adipose-derived stem cells were differentiated into endothelial cells and osteoblasts, and in vitro studies showed increased proliferation of cocultured cells compared with undifferentiated adipose-derived stem cells and monocultures of endothelial cells and osteoblasts. The cells were seeded into polylactic acid gas-plasma–treated scaffolds as cocultures and monocultures and then implanted into critical-sized rat calvarial defects. The cocultures were in a 1:1 osteoblast to endothelial cell ratio. The increase in proliferation seen by the cocultured cells in vitro did not translate to increased vascularization and osteogenesis in vivo. In vivo, there were trends of increased vascularization in the endothelial cell group and increased osteogenesis in the osteoblast and endothelial monoculture groups, but no increase was seen in the coculture group compared with the undifferentiated adipose-derived stem cells.

Endothelial cells enhance vascularization and osteoblast and endothelial cell monocultures enhance bone formation in the polymer scaffold. Predifferentiation of adipose-derived stem cells is promising for improving vascularization and osteogenesis in polymer scaffolds but requires future evaluation of coculture ratios to fully characterize this response.

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Received November 3, 2013.
Accepted for publication January 7, 2014.

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Supported by the National Endowment for Plastic Surgery and Plastic Surgery Foundation.
The authors report no conflicts of interest.

ISSN: 1049-2275
DOI: 10.1097/SCS.0000000000000755

Key Words: Adipose-derived stem cells, coculture, bone regeneration, tissue engineering, endothelial cell, osteoblast, rat calvarial defect, polylactic acid

(*J Craniofac Surg* 2014;25: 1504–1509)

Traumatic injuries can lead to significant morbidity if the injury involves bone defects that exceed the body's ability to repair itself to its preinjury state.[1] Bone autografts are the criterion standard for implant materials for critical-sized defects but are limited by availability of materials and donor-site morbidity.[2–4] Strategies for overcoming these shortcomings have involved synthetic or allogenic scaffolding with implantation of cells for bone regeneration.[5,6]

Adipose-derived stem cells (ASCs) have been investigated as a source of cells for implantation in scaffolds for bone regeneration.[7,8] They serve as a source of autologous stem cells that can be in adequate supply and are easily accessible compared with other sources of cells such as bone marrow or vessels. These cells are capable of differentiating into adipocytes, chondrocytes, osteoblasts (OBs), fibroblasts, and endothelial cells (ECs) [9–11] and have been successfully differentiated into ECs and OBs by our group.[5]

Because diffusion in a scaffold is limited to 100 to 150 μm,[12] it is important to not only regenerate bone but also allow for the development of a vascularized network to allow for the delivery of nutrients, growth factors, and oxygen to the newly developing bone. Seeding of undifferentiated ASCs or a single type of cell has been limited in forming a robust vessel network or adequate mineralization in large defects. Therefore, cocultures are being examined as a possible solution to this problem.

Coculture is the culture of cells together such as ECs with OBs. During the natural regeneration of bone, there are multiple synergistic interactions between these 2 types of cells.[13–16] Coculture studies have shown that ECs accelerate bone formation via angiogenesis and production of osteogenic factors, whereas OBs produce angiogenic factors such as vascular endothelial growth factor and matrix components.[16–18] These cocultures can be easily seeded within a polymeric scaffold.

Polylactic acid polymer scaffolds have the advantage of being degradable, easily moldable, and adequate in supply but are limited by the hydrophobicity of the surface.[19] Gas plasma treatment is a method of sterilization and surface treatment for polymers that improve attachment and proliferation of cells by attaching oxygen radicals to the surface and making the surface more hydrophilic.[20,21] It has also been shown to enhance angiogenesis in subcutaneous mouse models.[22]

The objective of this study was to implant gas-plasma–treated poly (D,L-lactide) polymer (PLA) scaffolds, seeded with either cocultures or monocultures of ASCs predifferentiated into ECs or OBs, into a critical-sized calvarial defect and assess bone regeneration and vessel formation. It is hypothesized that there will be a

greater amount of bone volume and vasculogenesis in the defects implanted with scaffolds containing cocultured cells.

MATERIALS AND METHODS

Adipose-Derived Stem Cell Isolation and Culture

The study protocol was approved by the University of Texas Health Science Center at San Antonio animal use committee. Isolation and differentiation of the ASCs were performed as done in previous studies by our group.[5] The ASC differentiation was based on protocols established by Zuk et al for endothelial differentiation.[9] Confirmation ASC differentiation to ECs was previously done by our group,[5,8] which included immunohistochemistry with CD31, von Willebrand Factor (VWF) western blot, angiogenic assays, and histologic assay. The same protocols were used for this study. Osteogenic differentiation of ASCs has also been confirmed in our previous studies with immunohistochemistry with osteocalcin as well as osteopontin, and the ability for calcium deposition was demonstrated with von Kossa staining.[8]

Under general anesthesia using isoflurane, adipose tissue excised from the perinephric and peritesticular fat pad of 2-month-old Lewis rats was placed in sterile pH-balanced Hank's balanced salt solution containing 1% bovine serum albumin (BSA) for further processing. The adipose tissue was finely minced using sharp dissection and centrifuged for 5 minutes at 500 × g. The free-floating fat was removed, and the remaining tissue/cell suspension was placed into 25 mL of 1% BSA Hank's balanced salt solution containing collagenase (200 U/mL) and allowed to agitate at 37°C for 1 hour. The digest was filtered through 100-μm nylon mesh twice and then centrifuged for 5 minutes at 500 × g. The supernatant was removed and the pellet was resuspended in MesenPro media (Invitrogen, Carlsbad, CA) to a concentration of 4×10^4 cells per milliliter and plated overnight and incubated at 37°C with 5% CO_2. Cells were used for differentiation after the third passage.

Osteogenic Induction

After the third passage, the ASCs were cultured in an osteogenic differentiation medium consisting of Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% penicillin/streptomycin, 0.1-μM dexamethasone, 10-μM β-glycerol phosphate, and 50-μM ascorbic acid. The osteogenic medium was changed every third day for 21 days.

Endothelial Induction

After the third passage, the ASCs were cultured in a vasculogenic medium consisting of Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% penicillin/streptomycin, and 50-ng/mL recombinant rat vascular endothelial growth factor-C (Promocell, Heidelberg, Germany). The media was changed every other day for 8 days before implantation in the bone allografts. The vascular endothelial growth factor-C was added fresh to stock media before each media change.

Immunocytochemistry

After the cells were cultured within the scaffolds for 21 days, the cells were fixed with 4% (w/v) paraformaldehyde, washed with phosphate-buffered saline (PBS; pH, 7.4), and permeabilized with PBS containing 0.2% Tween-20 for 30 minutes. The cells were then washed thrice with PBS and blocked with PBS containing 1% BSA and 10% goat serum for 1 hour at room temperature (RT) on a shaker. Primary antibody to osteocalcin was then added in blocking solution (sc-23790; Santa Cruz Biotech, Santa Cruz, CA), and the cells were kept at 4°C overnight on the shaker. After washing the next day, secondary antibody conjugated to Alexa 488 fluorochrome (A11055, Invitrogen, Carlsbad, CA) was added for 1 hour at RT. The cells were washed again as well as blocked for 30 minutes at RT, and primary antibody to VWF (sc-14014; Santa Cruz Biotech) was added to cells at 4°C overnight. The cells were washed again, and the secondary antibody conjugated to Alexa 568 (A11036 or A11004; Invitrogen) was added for 1 hour at RT, along with Hoechst 33258 (Invitrogen). The cells were then washed with PBS and rinsed in water, and the cover slips were transferred to a slide with an antifade (VectaShield; Vector Laboratories, Burlingame,CA), sealed with nail polish, and visualized in an Olympus FV1000 confocal microscope (Olympus, Center Valley, PA). Images were acquired sequentially and analyzed using FV10-ASW version 2.1 at 40-fold oil immersion magnification.

Preparation of PLA Scaffolds

The PLA scaffolds were fabricated using a vibrating particle salt-leaching method, which yields an open cell construct with interconnected pores, porosity of 90%, and pores 250 to 600 μm in diameter. Poly (D,L-lactic) polymer (DURECT Corp, Birmingham, AL) was dissolved in acetone, and the polymer solution was added to sodium chloride and vibrated under continuous flow conditions. Eight-millimeter–diameter disks of 1.2-mm thickness were punched out of the polymer mixture after drying, and the sodium chloride was leached out in sterile deionized water. After 2 days in water, the scaffolds were lyophilized. Before seeding, gas-plasma treatment was performed in a pure oxygen environment in a glow discharge system (PDC-32G, Plasma Cleaner/Sterilizer; Harrick Scientific, Inc, Pleasantville, NY) for 3 minutes at 100 W as a surface treatment and for sterilization.

Seeding PLA Scaffolds

Before seeding, the scaffolds were degassed in culture media under vacuum and placed in a 24-well ultralow attachment tissue culture plate (Corning Life Sciences, Lowell, MA). The cells were resuspended to a concentration of 100,000 cells per 20 μL before being added dropwise to the scaffold surface. The seeding efficiency of this method is approximately 80% based on our studies. The cells were allowed to incubate for 30 minutes at 37ºC 5% CO_2 before adding 500 μL of the culture media to each well.

Proliferation Assay

Proliferation of ASC, ECs, OBs, and cocultured ECs and OBs in the scaffolds was measured on days 1, 3, 7, 14, and 21 using the AlamarBlue (Invitrogen, Carlsbad, CA) assay. At each time point, the scaffolds were incubated for 1.5 hours at 37°C with 5% CO_2 in 500 μL of a 10% AlamarBlue solution in a new 24-well plate. After the incubation, the scaffolds were removed from the well plates, rinsed with PBS, transferred to a new plate with new media, and placed back in the incubator for further culture. Fluorescence was measured using a fluorescence plate reader at excitation at 540 nm and emission at 590 nm (n = 3).

Preparation of In Vivo Implant Samples

The PLA scaffolds were degassed and seeded in the same manner as that for the in vitro samples with a total of 100,000 cells per scaffold. The scaffolds were divided into 4 groups: (1) undifferentiated ASC (n = 8), (2) adipose tissue–derived OBs (Osteo, n = 5), (3) adipose tissue–derived ECs (Endo, n = 7), and (4) adipose tissue–derived EC and OB coculture in a 1:1 ratio (Osteo-Endo, n = 8).

In Vivo Assay

Two-month-old Lewis rats were anesthetized with isoflurane, and the scalps were shaved and prepared. The rat calvarias were exposed using a midline scalp incision, and the skin and periosteum reflected laterally. An 8-mm defect was created using an 8-mm trephine burr, leaving the dura mater intact. The critical-sized calvarial defects were treated with the previously seeded PLA scaffolds. The implants were secured in place with silk suture, and the scalp was closed with staples. At 8 weeks postoperatively, the rats were euthanized and their calvaria were harvested for radiographic and histologic examination.

Microcomputed Tomographic Analysis

The samples were scanned using microcomputed tomography (μCT) SkyScan 1076 (Skyscan, Aartselaar, Belgium) at 100-kV source voltage and 100-μA source current with no filter used and at a spatial resolution of 8.77 μm. The reconstructions were performed using NRecon software (Skyscan, Aartselaar, Belgium) and resulted in grayscale images that spanned a density range from 0.96 to 2.57 g/cm^3 corresponding to grayscale values of 0 to 255. DataViewer (Skyscan, Aartselaar, Belgium) was used to reslice the μCT images along coronal and sagittal axes, which were used to reorient the μCT slices to be perpendicular to the cranial-caudal axis of the calvaria, and μCT thresholding was performed to include only ossified tissues. The thresholds were selected as the histogram minima between the peaks representing the formalin in which the sample was scanned and the bone volume of the sample. The geometric mean of the threshold for all 100 samples in the study was used to determine the upper and lower threshold for binarization as 32 and 250. The region of interest (ROI) was chosen by creating a volume that spanned 8 mm around the defect generated in the calvaria and repaired with allograft to include the entire defect generated. Additional analyses were performed by choosing concentric ROI of 7 mm in diameter and the remainder of the native calvaria. All ROIs comprised the full thickness of the calvaria. The total volume of bone ingrowth in this three-dimensional volume was computed using CTAn software (Skyscan, Aartselaar, Belgium), and the mean bone density of the ossified tissues within these three-dimensional volumes was compared.

Histologic Evaluation of Vascularization

After μCT, the specimens were fixed in 10% neutral phosphate-buffered solution formalin, demineralized in EDTA solution, dehydrated in a series of alcohol washes, and embedded in paraffin. The specimens were sectioned in the coronal plane in the center of the bone scaffolds at a thickness of 6 μm and stained with hematoxylin and eosin for visualization through conventional qualitative bright-field microscopy analysis. For the quantification of blood vessels, 3 sections per animal stained with a rat-specific anti-CD34 antibody (R&D Systems AF4117/Goat pAb) and counterstained with hematoxylin were analyzed. Microscopic pictures were randomly taken at 10-fold magnification including 3 central and 3 peripheral areas per section for a total of 6 pictures. To determine the microvascular density (mean number of capillaries per square millimeter), the number of structures with lumen surrounded by CD34-positive cells was counted manually. Histomorphometric analysis of the tissue area on the pictures was performed using Image J (National Institutes of Health, Bethesda, MD). Image collection and quantification were performed by 1 author blinded to the treatment groups.

Statistical Analysis

The Kruskal-Wallis 1-way analysis of variance on ranks was used for statistical analysis using Statistical Analysis Software (SAS Institute, Cary, NC) and was performed for all acquired μCT and microvessel quantification data. All data are presented as the mean ± standard deviation. In all statistical evaluations, $P < 0.05$ was considered as statistically significant.

RESULTS

Endothelial Differentiation

Successful differentiation of ASCs into ECs for this experiment was confirmed using immunocytochemistry analysis. There was an increase in VWF expression of EC-differentiated ASC compared with nondifferentiated ASCs after 8 days of differentiation in endothelial differentiation media (Fig. 1).

Osteogenic Differentiation

Osteocalcin was prominently expressed on ASCs differentiated in osteogenic media for 21 weeks as shown by prominent expression of osteocalcin by the cells using immunofluorescence (Fig. 1).

Proliferation in PLA Scaffolds

There was increased proliferation of cells at all time points in scaffolds seeded with cocultured ECs and OBs (Osteo-Endo) compared with undifferentiated ASCs and monocultured EC (Endo) and OBs (Osteo). At day 21, there was a significantly higher amount of cells in the Osteo-Endo group compared with that in the other groups. Undifferentiated ADSCs had the lowest amount of proliferation at all time points. The amount of fluorescence at day 21 for the Osteo-Endo group was 11.112 ± 0.083 compared with 6.172 ± 0.080 (ASC), 7.081 ± 0.046 (Endo), and 9.093 ± 0.118 (Osteo) (Fig. 2).

Osteogenesis in PLA Scaffolds

Quantification of Bone Volume

Quantification of bone volume was performed using μCT analysis. The bone volume was measured in the 8-mm–diameter defect and also the 7-mm–diameter defect, which excludes the outer 0.5 mm of the defect to account for migration of native OBs and bone formation due to those cells. For the 8- and 7-mm ROIs, there was no statistically significant difference in bone volume, but there was an increased trend of increased bone volume in the Osteo group (Fig. 3).

Quantification of Bone Mineral Density

Bone mineral density was measured in gram hydroxyapatite per milliliter in the 8-mm defect 8 weeks after the implantation. The observed mineral density ranged from 1.132 to 1.309 mg HA/

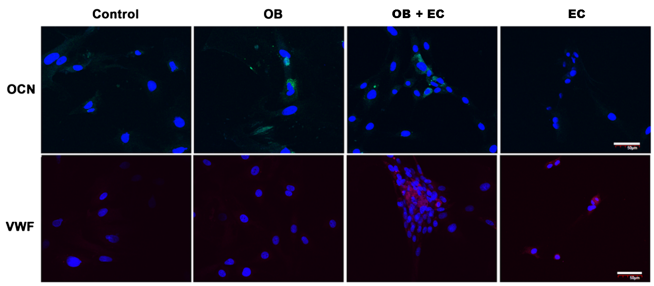

FIGURE 1. Immunofluorescence images of scaffolds seeded in vitro for 21 days. Blue indicates Hoechst = nucleus; green, OCN; red, VWF (40× oil-immersion magnification). OCN, osteocalcin.

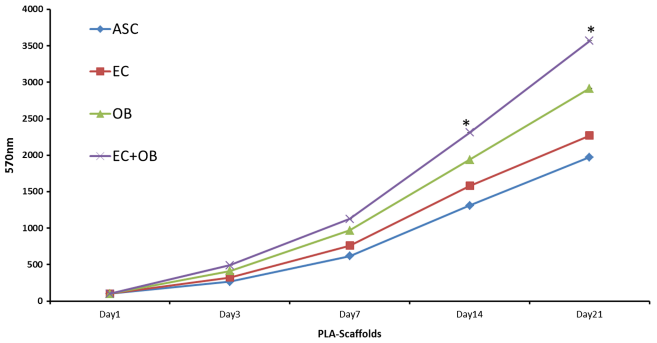

FIGURE 2. Cell proliferation: Cocultured ECs and OBs had significantly increased proliferation over 21 days compared with monocultured cells. The asterisk indicates $P < 0.05$).

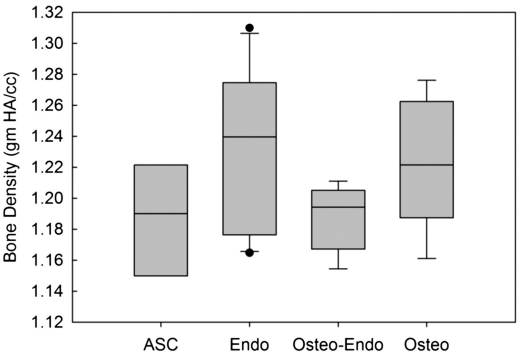

FIGURE 4. Bone mineral density measured in cubic millimeters within the 8-mm scaffold 8 weeks after the implantation. The results are presented as median as well as 25% and 75% quartiles with outliers indicated by black dots.

mL with the Endo and Osteo groups, showing increased trend of bone density compared with the ASC and Osteo-Endo groups. There was no statistically significant difference between the groups using Kruskal-Wallis 1-way analysis of variance ($P = 0.151$) (Fig. 4).

Vasculogenesis in PLA Scaffolds

Microvessel density was measured as vessel area/tissue area (%) in the 8-mm defect 8 weeks after the implantation. The median observed microvessel density ranged from 0.932% for the Endo group, 0.817% for the ASC group, 0.744% for the Osteo group, and 0.899% for the Osteo-Endo group, with the Endo group showing a slightly increased trend of increased microvessel density compared with the ASC, Osteo, and Osteo-Endo groups. There was no statistically significant difference between the groups ($P = 0.619$). The Kruskal-Wallis 1-way analysis of variance on ranks was used for statistical analysis (Fig. 5).

DISCUSSION

The PLA scaffolds were engineered to have porosity, interconnectivity, and surface morphology favorable to EC attachment and bone formation.[23,24] In addition, the gas-plasma treatment of the PLA scaffold has been shown to increase vascularization.[22] For these reasons, PLA scaffolds were used for this study and it was hypothesized that the use of PLA scaffolds with cocultured differentiated ASCs would result in improved vasculogenesis and osteogenesis within a critical-sized rat calvarial defect.

Although increased proliferation of cocultured cells was seen in vitro, this did not translate in vivo. There were no statistically significant differences seen between the 4 groups for vascularization or osteogenesis, but there were trends of increased vascularization in the Endo group and increased osteogenesis in the Osteo and Endo groups but none in the coculture group.

In previous studies from our group using allografts and PLA scaffolds in the same rat calvarial defect model using monocultured differentiated and undifferentiated ASCs, it was shown that there was an increase in vascularization when ECs were used in both the allograft and PLA implants, but bone formation was different between the 2 materials.[5,8] The PLA scaffolds displayed increased bone formation with OB seeding, and allografts had increased bone formation with endothelial seeding. The differences seen in these studies are thought to be directly caused by the material differences.

Endothelial cell and OB cocultures have been shown to have a synergistic effect between the 2 cell types, but as with other cell cultures, the results are dependent on the environment in which they are cultured.[25] In this study, the cocultured group had increased proliferation in vitro compared with the monocultured and undifferentiated culture groups, which did not translate into increased angiogenesis or osteogenesis in vivo. The limitation of the in vitro proliferation study is that it reflects the total number of cells and does not give a distribution of ECs to OBs present once the culture starts. Factors such as cell density, distribution, and timing of seeding within the scaffold contribute to formation of bone and vessels.[25,26]

Cocultured ECs and OBs were used in the allograft study, and it was believed that the lack of adequate porosity of the allograft and the use of the 1:1 coculture ratio were responsible for the limitations of the vasculogenic and osteogenic response. For

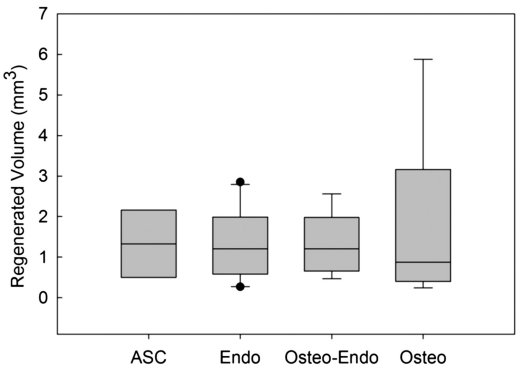

FIGURE 3. Bone volume quantification measured in cubic millimeters within the 8-mm scaffold 8 weeks after the implantation. The results are presented as median as well as 25% and 75% quartiles with outliers indicated by black dots.

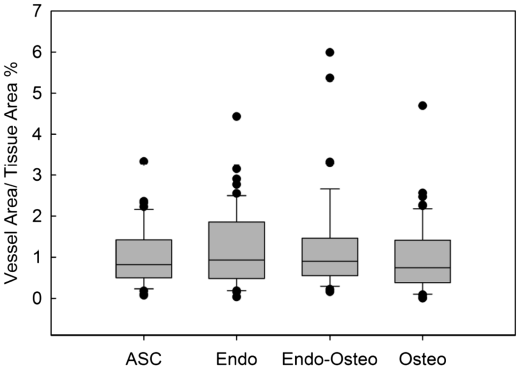

FIGURE 5. Microvessel density measured in cubic millimeters within the 8-mm scaffold 8 weeks after the implantation. The results are presented as median as well as 25% and 75% quartiles with outliers indicated by black dots.

this study, the 1:1 ratio was used again so that the results from the PLA scaffold seeding could be compared directly with the allograft response from the prior study to determine the impact of the scaffold material. The allograft study did show improved bone formation in the Endo group but not in the cocultured group likely because of the improved vascularization of the scaffold from the ECs, which would improve the diffusional and nutrient delivery profile. In addition, the ratio of ECs to OBs cultured was in a 1:1 ratio, which has been demonstrated to be effective in vitro.[27] Other studies demonstrated improved bone formation, however, with higher OB to EC ratios.[28] Despite the different scaffold materials used, there was no improvement in vascularization or mineralization seen with the use of cocultures, leading us to believe the decreased response is caused by the coculture ratio used. Implanted ratios of cocultured ECs and OBs have been manipulated to result in increased angiogenesis and/or mineralization.[27] By altering the ratio of the cocultured cells other than the 1:1 ratio, a culture could be pushed toward mineralization or vascularization.[29] In vitro, it has been seen that, in cocultures with more OBs than ECs, the cells will start to segregate with OBs forming bone mineralization nodules with surrounding ECs.[15] In the in vivo assay, there may have not been enough OBs in the cocultured group to segregate and form bone mineralization nodules within central areas of the scaffold. Most bone volume in the Osteo group comes from the periphery of the scaffold. Migrating cells from the neighboring native bone into the scaffold may have supplied the increased concentrate on of osteoblastic cells, leading to more bone formation. Osteoblasts have been shown to have increased migration when cultured with ECs,[30] which may explain why the Endo group was able to have bone formation similar to that of the other groups despite not having osteogenic cells seeded within the scaffold (Fig. 6).

In the Osteo in vivo group, the seeded cell density of osteogenic cells is higher, which led to a trend of higher bone volume and higher bone mineral density. There was no statistically significant difference, but the bone volume and bone mineral density results had high variability and, therefore, a chance for a type 2 statistical error.

Endothelial cells in coculture have been shown to have increased survival in prolonged cultures.[29,31] It has been postulated that this prolonged survival is caused by decreased proliferation and increased vessel formation because angiogenesis will not occur unless there is a senescence of the cells in order for tubule formation and branching to occur.[14] In the histologic analysis, there is a slight increase in the trend vessel formation seen in the defects for the Endo group and the trend of increased bone mineral density seen in the Endo group. The increase in vessel formation would lend itself to improved bone density in a small specific area that benefits from increased oxygen and nutrient delivery. However, without significant vessel formation throughout the scaffold, there would not be an increase in total bone volume.

In our series of 3 experiments,[5,8] it has been shown that vascularization and osteogenesis in a critical-sized rat calvarial defect can be improved with the use of allogenic bone and PLA scaffolds seeded with predifferentiated ASCs. The PLA scaffolds have been shown to support both vasculogenesis and osteogenesis, but lack the material properties to induce increased bone formation without the addition of OBs. In theory, the coculture of ECs and OBs should result in an increase in bone formation and vessel formation, but as this study shows, coculture does not always lead to increased bone formation in vivo. Coculture of the differentiated ADCs in vitro does result in increased total cell proliferation, which is greater than ECs or OBs alone, so there is a synergistic effect between the 2 cells, but this increase in proliferation has to be further explored to use the increase in cells to increase bone and vessel formation. In vivo, a trend of increased bone volume is seen when the scaffolds are seeded with OBs. There have been in vitro studies that show that manipulation of coculture cell ratios can help push cells into specific tissue formation. Future work exploring these cell ratios in vivo will reveal the full potential of the cocultures in polymer scaffolds.

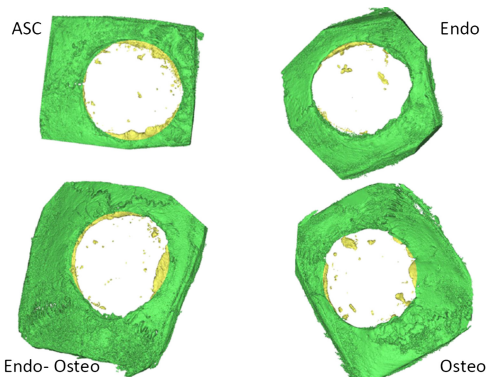

FIGURE 6. Microcomputed tomographic reconstructions of analyzed rat calvarial defects with implanted PLA scaffolds. Green indicates native bone; yellow, new bone.

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